Protease Inhibitors: Synthesis of Potent Bacterial Collagenase and Matrix Metalloproteinase Inhibitors Incorporating *N*-4-Nitrobenzylsulfonylglycine Hydroxamate Moieties

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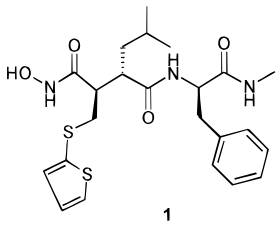
A series of compounds was prepared by reaction of alkyl/arylsulfonyl halides with *N*-4-nitrobenzylglycine, followed by conversion of the COOH to the CONHOH group, with hydroxylamine in the presence of carbodiimides. Other structurally related compounds were obtained by reaction of *N*-4-nitrobenzylglycine with aryl isocyanates, arylsulfonyl isocyanates, or benzoyl isothiocyanate, followed by the similar conversion of the COOH into the CONHOH moiety. Another subseries of derivatives was prepared from sulfanilyl- or metanilyl-4-nitrobenzylglycine by reaction with arylsulfonyl isocyanates, followed by conversion of the COOH to the hydroxamate moiety. The new compounds were assayed as inhibitors of four matrix metalloproteinases (MMPs), MMP-1, MMP-2, MMP-8, and MMP-9, and of the *Clostridium histolyticum* collagenase (ChC). Some of the prepared hydroxamate derivatives proved to be very effective collagenase/gelatinase inhibitors, depending on the substitution pattern at the sulfonamido moiety. Substitutions leading to best inhibitors of MMP-1, a short pocket enzyme, were those involving pentafluorophenylsulfonyl or 3-trifluoromethylphenylsulfonyl moieties at $P_{1'}$ (K_I's of 3−5 nM). For MMP-2, MMP-8, and MMP-9 (deep-pocket enzymes), best inhibitors were especially those containing long perfluoroalkylsulfonyl and substituted-arylsulfonyl moieties, such as pentafluorophenylsulfonyl, 3- and 4-protected-aminophenylsulfonyl, 3- and 4-carboxyphenylsulfonyl, arylsulfonylureido, or arylsulfonylureidosulfanilyl/metanilyl moieties, at $P_{1'}$. Bulkier groups in this position, such as 1- and 2-naphthyl, substituted-naphthyl, or quinolin-8-yl moieties among others, led to less effective MMP/ChC inhibitors. Best ChC inhibitors were again those containing pentafluorophenylsulfonyl or 3- and 4-protected-aminophenylsulfonyl $P_{1'}$ anchoring groups, suggesting that this protease is also a short-pocket wider-neck one (more similar to MMP-1). This study also proves that the 4-nitrobenzyl moiety is an efficient $P_{2'}$ anchoring moiety and that sulfonylureido, ureido, or carboxythioureido substitutions at $P_{1'}$ are also tolerated for obtaining potent sulfonylated amino acid hydroxamate-like MMP/ChC inhibitors.

Introduction

Matrix metalloproteinases (MMPs) have recently become interesting targets for drug design,[1−5] in the search of novel types of anticancer, antiarthritis, or other pharmacological agents useful in the management of inflammatory processes.[1−5] All these conditions are generally associated with enhanced activity of several zinc endopeptidases, of which the different MMPs actually known (20 such enzymes were reported for the moment in higher vertebrates, Table 1)[1−6] are responsible for the efficient degradation of all components of the extracellular matrix (ECM) at physiological pH values. ECM turnover is thus involved in crucial physiological and physiopathological events, such as embryonic development, blastocyst implantation, nerve growth, ovulation, morphogenesis, angiogenesis, tissue resorption and remodeling (such as in the case of wound healing), bone remodeling, apoptosis, cancer invasion and metastasis, arthritis, atherosclerosis, aneurysm, breakdown of blood−brain barrier, periodontal disease, skin ulceration, corneal ulceration, gastric ulcer, and liver fibrosis among others.[1−6] It is thus possible to envisage many medicinal chemistry applications by inhibiting the activity of these enzymes, and several pharmacological agents of the hydroxamate type, such as batimastat **(1)**, marimastat **(2)**, trocade **(3)**, or AG 3340, **(4)**, are currently in advanced clinical trials as antimetastasis, anticancer, antiarthritis drugs or as agents for the control of recurrant corneal growth (batimastat).[1−4,6,7]



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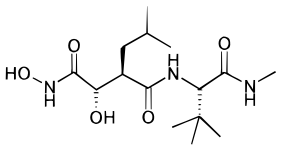

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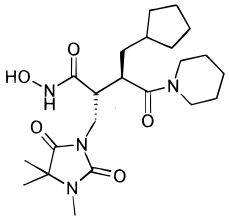

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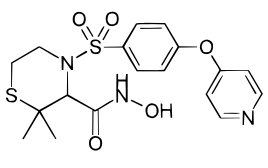

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Table 1. Vertebrate MMPs and Their Molecular Weights, Substrates, and Preferred Scissile Amide Bonds

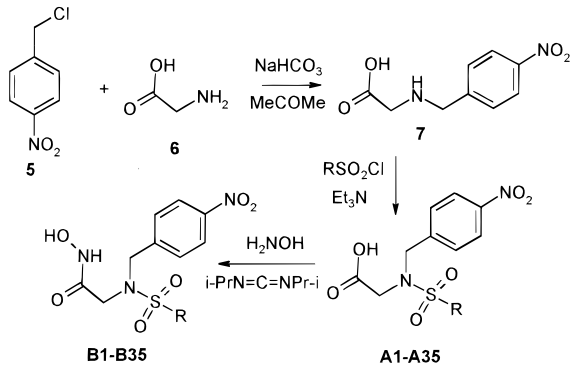
protein	MMP	MW (kDa)	principal substrate(s)	preferred scissile amide bond(s)
collagenase 1	MMP-1	52	fibrillar and nonfibrillar collagens (types I, II, III, VI, X), gelatins	Gly-Ile
gelatinase A	MMP-2	72	basement membrane and nonfibrillar collagens (types IV, V, VII, X), fibronectin, elastin	Ala-Met
stromelysin 1	MMP-3	57	proteoglycan, laminin, fibronectin, collagen (types III, IV, V, IX), gelatins, pro-MMP-1	Gly-Leu
matrilysin	MMP-7	28	fibronectins, gelatins, proteoglycan	Ala-Ile
collagenase 2	MMP-8	64	fibrillar collagens (types I, II, III)	Gly-Leu, Gly-Ile
gelatinase B	MMP-9	92	basement membrane collagens (types IV, V), gelatins	Gly-Ile, Gly-Leu
stromelysin 2	MMP-10	54	fibronectins, collagen (types III, IV), gelatins, pro-MMP-1	Gly-Leu
stromelysin 3	MMP-11	45	serpin	Ala-Met
macrophage elastase	MMP-12	53	elastin	Ala-Leu, Tyr-Leu
collagenase 3	MMP-13	51.5	fibrillar collagens (types I, II, III), gelatins	Gly-Ile
MT1-MMP	MMP-14	66	pro-72-kDa gelatinase	not determined
MT2-MMP	MMP-15	61	not determined	not determined
MT3-MMP	MMP-16	55	pro-72-kDa gelatinase	not determined
MT4-MMP	MMP-17	58	not determined	Ala-Gly
collagenase 4 (*Xenopus*)	MMP-18	53	not determined	Gly-Ile
RASI 1	MMP-19	?	gelatin	not determined
enamelysin	MMP-20	?	amelogenin (dentine), gelatin	not determined
XMMP (*Xenopus*)	MMP-21	?	not determined	not determined
CMMP (chicken)	MMP-22	?	not determined	not determined
(no trivial name)	MMP-23	?	not determined	not determined

As shown by the literature data,[8−10] the MMP inhibitors have been extensively studied in the last 15 years, to find medicinal chemistry applications. However, the same situation is not true for the inhibitors of other enzymes that degrade ECM, such as the bacterial collagenases (for instance the enzyme isolated from *Clostridium histolyticum*), which were much less investigated. This collagenase (EC 3.4.24.3) is a 116-kDa protein belonging to the M31 metalloproteinase family,[11,12] which is able to hydrolyze triple helical regions of collagen under physiological conditions, as well as an entire range of synthetic peptide substrates. In fact the crude homogenate of *C. histolyticum*, which contains several distinct collagenase isozymes,[11,12] is the most efficient system known for the degradation of connective tissue, being also involved in the pathogenicity of this and related clostridia, such as *C. perfringens*, which causes human gas gangrene and food poisoning among others.[13] Typically, these bacteria (and their collagenases) cause so much damage so quickly that antibiotics are ineffective. Thus, development of inhibitors against these collagenases is also an interesting target for drug design.

Similarly to the vertebrate MMPs, *C. histolyticum* collagenase (abbreviated as ChC) has the conserved HExxH zinc-binding motif, which in this specific case is His^{415}ExxH, with the two histidines (His 415 and His 419) acting as Zn(II) ligands, whereas the third ligand seems to be Glu 447, and a water molecule/hydroxide ion acting as nucleophile in the hydrolytic scission.[14,15] Similarly to the MMPs, ChC is also a multiunit protein, consisting of four segments, S1, S2a, S2b, and S3, with S1 incorporating the catalytic domain.[14] Although the two types of enzymes mentioned above (the MMPs and the bacterial ChC) are relatively different, it is generally considered that their mechanism of action for the hydrolysis of proteins and synthetic substrates is rather similar.[11−15] One must also mention that ChC could not be crystallized at the moment, so that X-ray crystallographic data are not available for the design of powerful inhibitors.

In this paper we report the preparation of a series of MMP and ChC inhibitors incorporating alkyl/arylsulfonamidoglycine hydroxamate as well as arylsulfonylureido/arylureidoglycine hydroxamate moieties in their molecule. Some of the new compounds, assayed for the inhibition of purified collagenases (MMP-1 and MMP-8), gelatinases (MMP-2 and MMP-9) and ChC, showed high affinity for these enzymes (in the nanomolar range), behaving as some of the best MMP/ChC sulfonylated inhibitors reported up to now. SAR is also discussed for both MMP inhibition as well as bacterial collagenase inhibition with these new types of derivatives.

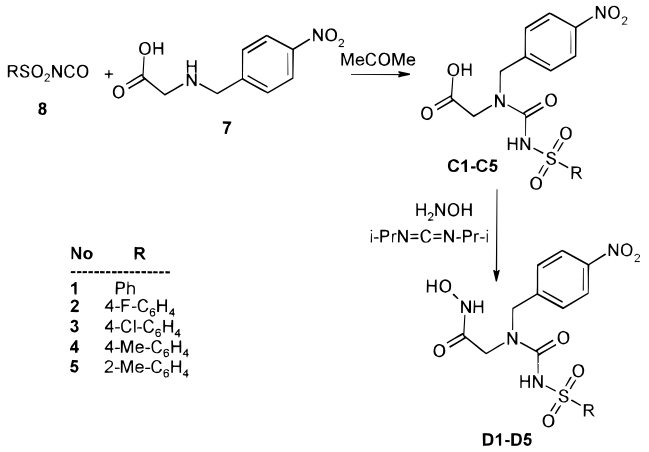
Scheme 1



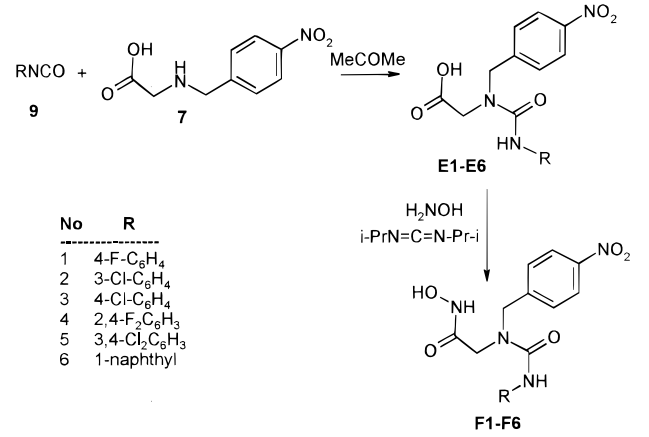
Results

Synthesis. Nonexceptional routine synthetic procedures were used for the preparation of the MMP/ChC inhibitors reported here, generally related to those applied by MacPherson et al.,[9a] Jeng et al.,[9b] Hanessian et al.,[9c] and Almstead's and Natchus' groups,[9d,e] for obtaining sulfonylated amino acid hydroxamates. Reaction of 4-nitrobenzyl chloride **(5)** with glycine **(6)** afforded the key intermediate *N*-4-nitrobenzylglycine **(7)**. Carboxylic acids **A1**−**A35** were then prepared by reaction of alkyl/arylsulfonyl chlorides with the glycine derivative **7** (Scheme 1). Conversion of the carboxylic acids **A1**−**A35** into the corresponding hydroxamates **B1**−**B35** was done with hydroxylamine and diisopropyl carbodiimide (Scheme 1).[16−19]

Scheme 2

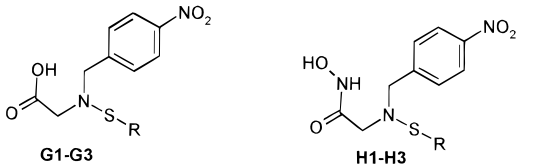


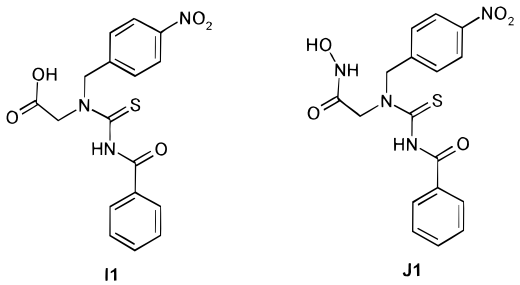
Scheme 3



Another series of derivatives (**C,D(1−5)**; **E,F(1−6)**) was obtained by reaction of arylsulfonyl isocyanates **8** or aryl isocyanates **9** with the *N*-benzylglycine derivative **7**, followed by the conversion of the COOH moiety into the CONHOH one, as described above (Schemes 2 and 3).[16−18,20]

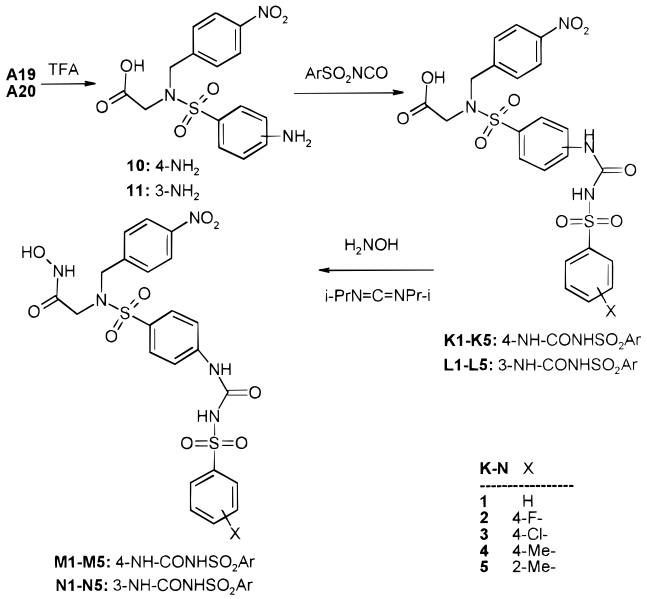
By applying synthetic strategies related to the previously described ones, the sulfenamido derivatives **G,H-(1−3)**, as well as the thioureas **I1** and **J1,** were also obtained.





Another approach for obtaining MMP and ChC inhibitors consisted in deprotection of the Boc derivatives

Scheme 4



A19 and **A20** (with TFA), followed by the reaction of the amino derivatives **10** and **11** with arylsulfonyl isocyanates, and conversion of the COOH into the hydroxamate moiety, leading thus to the carboxylates **K,L(1−5)** and the corresponding hydroxamates **M,N-(1−5)** (Scheme 4).

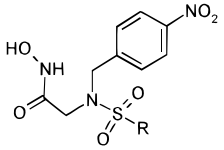
MMP and ChC Inhibitory Activity. Inhibition data against four MMPs (MMP-1, MMP-2, MMP-8, and MMP-9) and type II ChC with the compounds reported in the present paper are shown in Tables 2−4.

Discussion

Chemistry. Sulfonylated amino acid hydroxamates and sulfonylated amino acids (carboxylates) were recently discovered to act as efficient MMP inhibitors.[1,2,9,10a,19a] The most active compounds from this class of nonpeptide MMP inhibitors, of type **12**, possessed the following general structural features: (i) An arylsulfonyl group, occupying (but generally not filling) the specificity $S_{1'}$ pocket of the enzyme.[9,10a,19] It was also shown that the SO_2 moiety of the inhibitor is involved in several strong hydrogen bonds with amino acid residues from the active site cleft, which strongly stabilize the enzyme−inhibitor adduct.[9,10,19] (ii) Hydrogen or, better, an isopropyl substituent α to the hydroxamic/carboxylic acid moiety. The isopropyl moiety was considered to slow metabolism of the zinc-binding function.[1] By means of X-ray crystallography[3] and NMR spectroscopy[21] it was shown that this group binds within the S_1 subsite of the protease. It has also been demonstrated that this moiety can efficiently be replaced by bulkier groups (such as indolylmethyl in some tryptophan derivatives), without loss of MMP inhibitory properties.[19a] (iii) An isobutyl, pyridylmethyl, or benzyl moiety substituting the amino nitrogen atom, which binds within the $S_{2'}$ shallow and solvent-exposed pocket of the enzyme.[9,10,19a]

The promising MMP inhibition and preliminary clinical data of some of these sulfonamide inhibitors prompted us to investigate them in greater detail. Our lead compound was of type **12**, previously reported by MacPherson et al.,[9a] to which we performed the following structural modifications: (i) A large number of

Table 2. Inhibition of MMPs and ChC with the Hydroxamates **B1**−**B35**



B1-B35

		K_I [a] (nM)				
R	compd	MMP-1[b]	MMP-2[b]	MMP-8[b]	MMP-9[b]	ChC[c]
CH_3	**B1**	75	19	24	30	86
CF_3	**B2**	21	15	20	29	73
CCl_3	**B3**	24	17	19	25	70
n-C_4F_9	**B4**	62	1.5	2.4	2.0	12
n-C_8F_{17}	**B5**	79	0.9	1.3	1.3	8
Me_2N	**B6**	26	36	35	43	79
C_6H_5	**B7**	21	18	24	15	51
$PhCH_2$	**B8**	24	18	26	16	50
4-F-C_6H_4	**B9**	19	15	15	14	42
4-Cl-C_6H_4	**B10**	22	15	18	17	41
4-Br-C_6H_4	**B11**	25	13	21	21	36
4-I-C_6H_4	**B12**	29	19	17	16	32
4-CH_3-C_6H_4	**B13**	30	20	24	27	45
4-O_2N-C_6H_4	**B14**	15	9	7	8	13
3-O_2N-C_6H_4	**B15**	18	12	10	8	12
2-O_2N-C_6H_4	**B16**	59	15	13	14	18
3-Cl-4-O_2N-C_6H_3	**B17**	31	7	6	7	10
4-AcNH-C_6H_4	**B18**	14	8	9	12	10
4-BocNH-C_6H_4	**B19**	17	10	11	10	9
3-BocNH-C_6H_4	**B20**	26	11	15	14	9
C_6F_5	**B21**	3	0.7	0.1	0.6	5
3-CF_3-C_6H_4	**B22**	5	1.1	0.7	0.8	5
2,5-$Cl_2C_6H_3$	**B23**	9	10	13	17	16
4-MeO-C_6H_4	**B24**	28	18	21	31	20
2,4,6-$Me_3C_6H_2$	**B25**	36	25	29	39	24
4-MeO-3-BocNH-C_6H_3	**B26**	25	5	7	7	6
2-HO-3,5-Cl_2-C_6H_2	**B27**	12	12	15	16	9
3-HOOC-C_6H_4	**B28**[d]	41	3.0	3.3	2.5	10
4-HOOC-C_6H_4	**B29**[d]	34	2.5	2.4	2.8	7
1-naphthyl	**B30**	87	39	45	44	15
2-naphthyl	**B31**	62	33	43	50	10
5-Me_2N-1-naphthyl	**B32**	83	41	58	53	12
2-thienyl	**B33**	14	13	12	10	9

[a] K_I values were obtained from Easson−Stedman[23] plots using a linear regression program, from at least three different assays. Standard errors were of 5−10%. [b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically.[24] [c] With FALGPA as substrate, spectrophotometrically.[25] [d] The C_6H_4-COOH moiety transformed into C_6H_4-CONHOH.

diverse alkyl/arylsulfonyl, arylsulfonylureido/arylureido, or arylsulfenyl moieties were incorporated as $S_{1'}$ anchoring groups, since they seem to be the major determinants of activity/selectivity toward the different MMPs.[1−3,9,10,19] One has to mention that arylsulfonylureido/arylureido or arylsulfenyl moieties have not been previously incorporated in MMP inhibitors, probably because it has been reported[9a] that the carboxamido analogues of the sulfonamide hydroxamates **12** are inactive as MMP-3 inhibitors. (ii) The P_1 substituent (A in structure **12**) is absent in the compounds designed by us, which are all glycine derivatives. (iii) The already optimized[9] benzyl group at the $S_{2'}$ site has been modified to a 4-nitrobenzyl one, since we have previously shown that substituents at the benzyl moiety with acidifying properties, such as 2-nitro, 2-chloro, etc., lead to better ChC inhibitors as compared to the corresponding unsubstituted derivatives.[10c,22] This moiety has not been investigated previously as an $S_{2'}$ anchoring group,[9] may be also due to the fact that in the pioneering work of MacPherson et al.[9a] it was reported that the related 4-O_2N-$C_6H_4SO_2$ moiety (in $S_{1'}$) attached to *N*-isobutylglycine hydroxamate (compound **13**) led to an inactive MMP-3 inhibitor.

Table 3. Inhibition of MMPs and ChC with the Hydroxamates of Types **D**, **F**, **H**, and **J**

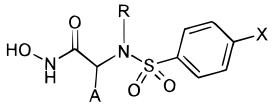
		K_I [a] (nM)				
R	compd	MMP-1[b]	MMP-2[b]	MMP-8[b]	MMP-9[b]	ChC[c]
Ph	**D1**	39	5	4	3	15
4-F-C_6H_4	**D2**	43	3	5	7	10
4-Cl-C_6H_4	**D3**	46	3	4	5	9
4-CH_3-C_6H_4	**D4**	54	6	6	8	12
2-CH_3-C_6H_4	**D5**	62	8	5	7	10
4-F-C_6H_4	**F1**	30	9	12	11	21
3-Cl-C_6H_4	**F2**	32	13	10	9	16
4-Cl-C_6H_4	**F3**	25	10	9	9	15
2,4-F_2-C_6H_3	**F4**	24	7	8	6	13
3,4-$Cl_2C_6H_3$	**F5**	31	6	5	7	11
1-naphthyl	**F6**	>100	35	39	33	18
4-O_2N-C_6H_4	**H1**	18	10	8	11	13
2-O_2N-C_6H_4	**H2**	24	12	13	14	10
2,4-$(O_2N)_2$-C_6H_3	**H3**	33	13	10	9	12
	J1	37	2	1.5	1.4	6

[a] K_I values were obtained from Easson−Stedman[23] plots using a linear regression program, from at least three different assays. Standard errors were of 5−10%. [b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically.[24] [c] With FALGPA as substrate, spectrophotometrically.[25]

Table 4. Inhibition of MMPs and ChC with the Carboxylates and Hydroxamates of Types **K**, **L**, **M**, and **N**

	K_I [a] (nM)				
compd	MMP-1[b]	MMP-2[b]	MMP-8[b]	MMP-9[b]	ChC[c]
K1	>100	18	23	21	24
K2	>100	16	21	23	29
K3	95	21	24	36	33
K4	94	24	28	25	30
K5	89	25	22	29	36
L1	>100	19	27	22	28
L2	>100	15	34	18	30
L3	>100	18	32	23	37
L4	>100	21	30	21	33
L5	>100	24	31	28	39
M1	61	0.6	0.7	0.9	15
M2	54	0.6	0.5	1.2	18
M3	50	0.8	1.1	1.0	13
M4	55	0.7	1.2	0.5	19
M5	72	1.6	1.5	0.7	20
N1	60	1.2	1.0	1.3	18
N2	49	1.8	1.4	2.5	17
N3	50	1.5	1.3	1.6	21
N4	53	2.5	2.1	3.1	20
N5	58	2.8	2.0	3.0	23

[a] K_I values were obtained from Easson−Stedman[23] plots using a linear regression program, from at least three different assays. Standard errors were of 5−10%. [b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically.[24] [c] With FALGPA as substrate, spectrophotometrically.[25]



12: A = H, i-Pr; R = i-Bu; Bn, etc
X = OMe; Cl; H; Me; F; Me_2N, etc

13: A = H; R = i-Bu; X = NO_2

The new compounds reported here were obtained by nonexceptional synthetic procedures, similar to those reported for structurally related compounds,[9] as outlined in Schemes 1−4. These involved reaction of *N*-4-nitrobenzylglycine **(7)** with alkyl/arylsulfonyl chlorides,[17] aryl isocyanates,[20] arylsulfonyl isocyanates,[20] or benzoyl isothiocyanate, followed by conversion of the

COOH moiety to the hydroxamate one.[16] Related synthetic strategies led to some sulfenamides of type **G** and **H**, as well as to the thioureas **I**,**J**. As it has recently been reported[1,9b,10] that elongated moieties in $P_{1'}$ may lead to selectivity of the obtained inhibitors for the deep pocket enzymes, relative to the short pocket ones, we have also studied an approach for preparing inhibitors containing such moieties (Scheme 4). Thus, the Boc-protected derivatives **A19** and **A20** were treated with TFA in order to eliminate the protecting group, and the amino derivatives thus obtained were reacted with arylsulfonyl isocyanates **8**. The carboxy groups of derivatives **K**,**L(1**−**5)** were then converted to the hydroxamate moieties by the standard procedure, as mentioned above, leading thus to derivatives **M**,**N(1**−**5)**.

MMP and ChC Inhibitory Activity. Inhibition data of Tables 2−4 show that the entire class of sulfonylated glycine hydroxamate derivatives reported here act as efficient metallopeptidase inhibitors, but important differences of activity were detected against the diverse enzymes, for the different substitution patterns of the alkyl/arylsulfonamide moiety contained in the molecules of the new inhibitors.

Thus, for MMP-1, an enzyme possessing a short specificity $S_{1'}$ pocket,[1] aliphatic (**B1**−**B6**, **B35**), bulky aromatic (**B25**, **B30**−**B32**, **D1**−**D5**, **F5**, **F6**, **J1**, **M,N-(1**−**5)**) or heterocyclic (**B34**) moieties as $P_{1'}$ groups led to the least effective hydroxamate inhibitors, with K_I's in the 30−80 nM range. The carboxylates **K**,**L(1**−**5)** were also ineffective in inhibiting this collagenase. Obviously, these data may be explained by the restricted space available around the $S_{1'}$ subsite of MMP-1, which cannot accommodate bulky groups in it, discriminating in this way between the bulky and less voluminous inhibitors. Inhibitors with K_I's in the 15−30 nM range against MMP-1 were also obtained. These contained aromatic rings, eventually substituted *meta* or *para* with electron-attracting groups such as nitro, halogeno, methoxy, etc. Some efficient MMP-1 inhibitors (K_I's in the range of 3−15 nM) were those containing the following moieties: 4-acetamidophenyl (**B18**), perfluorophenyl (**B21**), 3-trifluoromethylphenyl (**B22**), 2,5-dichlorophenyl (**B23**), or 2-hydroxy-3,5-dichlorophenyl (**B27**), among others.

The deep-pocket enzymes MMP-2, MMP-8, and MMP-9 showed a more similar inhibition behavior, relatively different from that of the previously discussed MMP-1. Thus, again short aliphatic moieties at $P_{1'}$ led to the least effective inhibitors, such as **B1**, **B6**, and **B35**. The same was true for the bulky naphthyl-derived moieties, such as in **B30**−**B32**, **B34**, and **F6**. All these derivatives possessed K_I's over 20 nM against these three MMPs. More effective inhibitors (K_I's in the range of 5−20 nM) were the aromatic derivatives, containing moieties such as 4-halogenophenyl, nitrophenyl, Boc-aminophenyl, etc. (**B9**−**B12**, **B14**−**B20**, **B23**, **B24**, **B27**, **D,F(1**−**5)**, **H1**−**H3,** and **J1**). Several of the new inhibitors prepared showed affinities in the 0.5−3.0 nM range. These were: (i) the perfluoroalkyl/ary-sulfonyl compounds **B4**, **B5**, and **B21/B22**; (ii) the carboxyphenyl-substituted derivatives **B28** and **B29**; and (iii) the elongated derivatives **M,N(1**−**5)**. Since the most active inhibitors contained perfluoroalkyl/arylsulfonyl moieties in their molecules, it is possible that F−H hydrogen bonds between the inhibitor and the active site ($S_{1'}$) residues are formed which contribute in stabilizing the E-I adduct, but this hypothesis should be verified by X-ray crystallography. One must mention that the same perfluorophenyl moiety induced very strong MMP-3 inhibitory properties when present in the thiadiazole urea inhibitors recently reported by Jacobsen et al.,[27] although these derivatives bind in a completely different manner to the enzyme (they are left-hand-sided inhibitors) than the arylsulfonylated amino acid hydroxamates/carboxylates investigated by us here and by other researchers.[9]

Inhibition of the bacterial collagenase ChC with the new compounds reported here generally paralleled the MMP-1 inhibition data. Potent inhibitors were obtained from all classes of hydroxamates investigated here, such as the alkyl/arylsulfonyl-4-nitrobenzyl-Gly derivatives (**B5**, **B20**−**B22**, **B26**, **B27**, **B29**, **B33**, **B34,** etc.), the arylsulfonylureas and arylureas (such as **D3**, **D5**, **F5**), the sulfenamido-4-nitrobenzyl-Gly derivatives (such as **H2**, **H3**), or the thiourea **J1**. It seems that the $S_{1'}$ binding moiety of the arylsulfonamide type, previously investigated for obtaining nonpeptide MMP inhibitors,[9] can be efficiently substituted by related moieties such as alkylsulfonyl, arylsulfenyl, arylsulfonylureido, arylureido, or benzoylthioureido without loss of the MMP/ChC inhibitory properties. In the subseries of alkyl/arylsulfonamido derivatives (of types **B**(**1**−**35**)) best ChC inhibitory properties were correlated with the presence of perfluoroalkylsulfonyl (**B4**, **B5**), perfluorophenylsulfonyl (**B21**), 3-trifluoromethylphenylsulfonyl (**B22**), 3-chloro-4-nitrophenylsulfonyl (**B17**), 3- or 4-protected-aminophenylsulfonyl (**B18**−**B20**, **B26**), and 3- or 4-carboxyphenylsulfonyl (**B28**, **B29**). All these derivatives possessed inhibition constants in the range of 5−10 nM against ChC. A second group of sulfonamide inhibitors, containing moieties such as 4-bromophenyl, 4-iodophenyl, 2-, 3-, or 4-nitrophenyl, 2,5-dichlorophenyl, 4-methoxyphenyl, or 2-thienyl, substituting the *N*-4-nitrobenzylglycine hydroxamate, behaved as medium potency inhibitors, with affinities in the 12−30 nM range. The least active sulfonamides were those containing methyl, trihalomethyl, dimethylamino, phenyl, and benzyl moieties. The arylsulfonylureido compounds **D1**−**D4** were more active than the corresponding arylsulfonyl derivatives (compare for instance **D1** with **B9**, **D2** with **B10**, etc.), acting as strong to medium potency ChC inhibitors. The ureas of type **F** and the sulfenamides of type **H** behaved similarly. A very potent inhibitor was the thiourea derivative **J1**. The elongated molecule compounds of types **K**−**N** were slightly less effective as compared to the previously mentioned derivatives. These data seem to indicate that ChC is similar to a short-pocket MMP, eventually possessing a slightly wider neck than MMP-1.

Conclusion

We describe here a novel class of strong inhibitors of the zinc proteases MMP-1, MMP-2, MMP-8, and MMP-9 and ChC (EC 3.4.24.3), a collagenase from *C. histolyticum*. The drug design has been realized by utilizing X-ray data for the adduct of some MMPs, with inhibitors of the sulfonyl amino acid hydroxamate/carboxylate type. Reaction of *N*-4-nitrobenzylglycine with sulfonyl chlorides, arylsulfonyl isocyanate, aryl isocyanates, or

benzoyl isothiocyanate afforded the glycine derivatives which were subsequently converted to the corresponding hydroxamates. Best substitutions for obtaining high-affinity inhibitors (0.5−2.5 nM) involved hydrophobic moieties at $S_{1'}$, such as perfluoroalkylsulfonyl, substituted-arylsulfonyl, pentafluorophenylsulfonyl, 3- and 4-carboxyphenylsulfonyl, and 3-trifluoromethylphenylsulfonyl, among others. It has also been proved that the arylsulfonyl anchoring $P_{1'}$ moiety can be replaced by alkylsulfonyl, arylsulfenyl, arylsulfonylureido, arylureido, or benzoylthioureido moieties, without loss of the MMP/ChC inhibitory properties. Some of the new inhibitors possessing elongated moieties at $P_{1'}$ also showed selectivity for the deep-pocket enzymes (MMP-2, MMP-8, and MMP-9) over MMP-1 and ChC. It seems that ChC (for which X-ray crystallographic data are not available for the moment) is a short-pocket enzyme, eventually possessing a wider neck than MMP-1.

Experimental Section

General. Melting points: heating plate microscope; not corrected. IR spectra: KBr pellets; 400−4000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer. ^1H NMR spectra: Varian Gemmini 200 apparatus; chemical shifts are expressed as δ values relative to Me_4Si as standard. Elemental analysis: ±0.4% of theoretical values, calculated for the proposed formulas for all the compounds reported here; Carlo Erba Instrument CHNS elemental analyzer, model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Preparative HPLC was performed on a Dynamax-60A column (25 × 250 mm), with a Beckman EM-1760 instrument. The detection wavelength was 254 nm.

Amino acids (Gly), 4-nitrobenzyl chloride, sulfonyl chlorides, arylsulfonyl isocyanates, aryl isocyanates, benzoyl isothiocyanate, triethylamine, carbodiimides, hydroxylamine, 5,5′-dithiobis(2-nitrobenzoic acid), FALGPA, buffers, and other reagents used in the syntheses were commercially available compounds, from Sigma, Acros, or Aldrich. The thioester MMP substrate AcProLeuGly-S-LeuLeuGlyOEt was from Bachem.

Preparation of *N*-4-Nitrobenzylglycine, 7. An amount of 7.5 g (0.10 M) of Gly **(6)** and the stoichiometric amount of 4-nitrobenzyl chloride (16.1 g) were suspended/dissolved in 150 mL of anhydrous acetonitrile and the equivalent amount of triethylamine (0.10 mM, 14.7 mL) was added. The reaction mixture was stirred at room temperature for 20 h, then the solvent was evaporated in vacuo. The obtained reaction mixture was taken in 250 mL of water, the pH was brought to 7 with citric acid, and the crude product **7** precipitated by leaving the mixture overnight at 4 °C. Recrystallization from ethanol afforded the pure title compound in almost quantitative yield.

General Procedure for the Preparation of *N*-4-Nitrobenzylalkyl/arylsulfonylglycines A1−A35. An amount of 2.10 g (10 mmol) of *N*-2-nitrobenzylglycine **(7)** and 10 mmol of sulfonyl chloride were suspended/dissolved in 100 mL of acetone + 25 mL of water. The stoichiometric amount (10 mmol) of base ($NaHCO_3$, $KHCO_3$, NaOH, or Et_3N) dissolved in a small amount (20 mL) of water was added and the mixture stirred at room temperature for 4−10 h (TLC control). The solvent was evaporated, the reaction mixture was retaken in 100 mL of water and the crude product extracted in ethyl acetate. After evaporation of the solvent, the compounds **A1−A35** were recrystallized from EtOH or MeOH. Yields were around 75−90%.

General Procedure for the Preparation of Compounds B1−B35, D1−D5, F1−F6, H1−H3, and J1. An amount of 5 mM of carboxylic acid derivative **A1−A35, C1−C5, E1−E6, G1−G3,** or **I1** was dissolved/suspended in 50 mL of anhydrous acetonitrile or acetone and treated with 420 mg (6 mM) of hydroxylamine·HCl and 1.10 g (6 mM) of EDCI·HCl or diisopropyl carbodiimide. The reaction mixture was magnetically stirred at room temperature for 15 min, then 180 μL (12 mM) of triethylamine was added and stirring was continued for 12 h at 4 °C. The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent removed in vacuo. Preparative HPLC (Dynamax-60A column (25 × 250 mm); 90% acetonitrile/10% methanol; flow rate of 30 mL/min) afforded the pure hydroxamic acids.

General Procedure for the Preparation of Compounds C1−C5, E1−E6, and I1. An amount of 2.10 g (10 mmol) of *N*-4-nitrobenzylglycine **(7)** and the stoichiometric amount of arylsulfonyl isocyanate **8**, aryl isocyanate **9**, or benzoyl isothiocyanate were suspended in 50 mL of anhydrous acetonitrile and 150 μL (10 mM) of triethylamine was added. The reaction mixture was either stirred at room temperature (in the case of derivatives prepared from **8**) or refluxed (for the other two types of derivatives) for 2−6 h. The solvent was evaporated and the reaction mixture worked up as described above. The new compounds were recrystallized from ethanol. Yields were almost quantitative.

General Procedure for the Preparation of Compounds G1−G3. The general procedure described above for the preparation of compounds **A1−A35** has been followed, except that arylsulfenyl halides were used instead of alkyl/arylsulfonyl halides. The yields in the title sulfenamides were around 75%.

General Procedure for the Preparation of Derivatives M,N(1−5). An amount of 10 mM of Boc-protected derivative, **A19** or **A20**, was treated with a small excess of TFA at room temperature, under energetic magnetic stirring, for 30 min. The excess acid was evaporated in vacuo, the residue taken in water and neutralized with sodium bicarbonate till pH 5.5. The precipitated amino derivatives **10** and **11** were filtered and air-dryed. Reaction of these derivatives with arylsulfonyl isocyanates **8** in acetone, as described above, afforded the carboxylates **K,L(1−5)** which were converted to the corresponding hydroxamates as described above. Overall yields were around 60−63%.

All the new compounds were characterized by ^1H and ^{13}C NMR spectroscopy and elemental analysis. Data for a representative compound of each series are provided below.

***N*-4-Toluenesulfonyl-*N*-4-nitrobenzylglycine, A13**: white crystals, mp 215−6 °C; ^1H NMR (DMSO-d_6) δ 2.50 (s, 3H, CH_3C$_6$H$_4$), 3.62 (s, 2H, CH_2 of Gly), 4.36 (s, 2H, CH_2 of benzyl), 7.25−7.64 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 7.97 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H_{meta} of $CH_3C_6H_4$), 8.21 (d, 2H, H_{meta} of $O_2NC_6H_4$), 11.69 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 26.0 (s, *C*H$_3$C$_6$H$_4$), 40.4 (s, *C*H$_2$ of Gly), 44.7 (s, *C*H$_2$ of benzyl), 123.8 (s, C_{meta} of $O_2NC_6H_4$), 129.4 (C_{ortho} of $O_2NC_6H_4$), 130.5 (s, C_{meta} of $CH_3C_6H_4$), 135.0 (s, C_{ortho} of $CH_3C_6H_4$), 144.5 (s, C_{ipso} of $O_2NC_6H_4$), 145.0 (s, C_{ipso} of $CH_3C_6H_4$), 147.8 (s, C_{para} of $O_2NC_6H_4$), 148.6 (s, C_{para} of $CH_3C_6H_4$), 177.3 (s, *C*O$_2$H). Anal. Found: C, 52.57; H, 4.76; N, 7.54. $C_{16}H_{16}N_2O_6S$ Requires: C, 52.74; H, 4.43; N, 7.69.

***N*-4-Toluenesulfonyl-*N*-4-nitrobenzylglycine hydroxamate, B13**: white crystals, mp 239−40 °C; ^1H NMR (DMSO-d_6) δ 2.62 (s, 3H, CH_3C$_6$H$_4$), 3.71 (s, 2H, CH_2 of Gly), 4.39 (s, 2H, CH_2 of benzyl), 7.21−7.67 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 8.00 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H_{meta} of $CH_3C_6H_4$), 8.21 (d, 2H, H_{meta} of $O_2NC_6H_4$), 8.74 (br s, 1H, N*H*OH), 10.51 (br s, 1H, NHO*H*); ^{13}C NMR (DMSO-d_6) δ 26.5 (s, *C*H$_3$C$_6$H$_4$), 40.6 (s, *C*H$_2$ of Gly), 45.1 (s, *C*H$_2$ of benzyl), 123.8 (s, C_{meta} of $O_2NC_6H_4$), 129.4 (C_{ortho} of $O_2NC_6H_4$), 130.7 (s, C_{meta} of $CH_3C_6H_4$), 135.6 (s, C_{ortho} of $CH_3C_6H_4$), 144.3 (s, C_{ipso} of $O_2NC_6H_4$), 145.3 (s, C_{ipso} of $CH_3C_6H_4$), 147.9 (s, C_{para} of $O_2NC_6H_4$), 148.6 (s, C_{para} of $CH_3C_6H_4$), 174.0 (s, *C*ONHOH). Anal. Found: C, 50.77; H, 4.39; N, 11.02. $C_{16}H_{17}N_3O_6S$ Requires: C, 50.65; H, 4.52; N, 11.08.

***N*-4-Toluenesulfonylureido-*N*-4-nitrobenzylglycine, C3**: white crystals, mp 237−8 °C; ^1H NMR (DMSO-d_6) δ 2.60 (s, 3H, CH_3C$_6$H$_4$), 3.67 (s, 2H, CH_2 of Gly), 4.38 (s, 2H, CH_2 of

benzyl), 7.29−7.73 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 7.99 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H_{meta} of $CH_3C_6H_4$), 8.16 (d, 2H, H_{meta} of $O_2NC_6H_4$), 8.29 (br s, 2H, NHCONH), 11.73 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 26.1 (s, $CH_3C_6H_4$), 40.8 (s, CH_2 of Gly), 45.3 (s, CH_2 of benzyl), 123.9 (s, C_{meta} of $O_2NC_6H_4$), 129.4 (C_{ortho} of $O_2NC_6H_4$), 131.9 (s, C_{meta} of $CH_3C_6H_4$), 132.4 (s, NH*C*ONH), 135.0 (s, C_{ortho} of $CH_3C_6H_4$), 144.5 (s, C_{ipso} of $O_2NC_6H_4$), 145.7 (s, C_{ipso} of $CH_3C_6H_4$), 147.8 (s, C_{para} of $O_2NC_6H_4$), 148.6 (s, C_{para} of $CH_3C_6H_4$), 177.3 (s, CO_2H). Anal. Found: C, 50.34; H, 4.08; N, 10.15. $C_{17}H_{17}N_3O_7S$ Requires: C, 50.12; H, 4.21; N, 10.31.

***N*-4-Toluenesulfonylureido-*N*-4-nitrobenzylglycine hydroxamate, D3**: white crystals, mp 273−4 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (s, 3H, $CH_3C_6H_4$), 3.69 (s, 2H, CH_2 of Gly), 4.36 (s, 2H, CH_2 of benzyl), 7.20−7.74 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 7.99 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H_{meta} of $CH_3C_6H_4$), 8.21 (d, 2H, H_{meta} of $O_2NC_6H_4$), 8.32 (br s, 2H, NHCONH), 8.76 (br s, 1H, N*H*OH), 10.59 (br s, 1H, NHO*H*); ^{13}C NMR (DMSO-d_6) δ 26.3 (s, $CH_3C_6H_4$), 40.9 (s, CH_2 of Gly), 45.2 (s, CH_2 of benzyl), 123.7 (s, C_{meta} of $O_2NC_6H_4$), 129.5 (C_{ortho} of $O_2NC_6H_4$), 131.3 (s, C_{meta} of $CH_3C_6H_4$), 132.4 (s, NH*C*ONH), 135.0 (s, C_{ortho} of $CH_3C_6H_4$), 144.5 (s, C_{ipso} of $O_2NC_6H_4$), 145.6 (s, C_{ipso} of $CH_3C_6H_4$), 147.7 (s, C_{para} of $O_2NC_6H_4$), 148.6 (s, C_{para} of $CH_3C_6H_4$), 174.6 (s, *C*ONHOH). Anal. Found: C, 48.09; H, 4.43; N, 13.12. $C_{17}H_{18}N_4O_7S$ Requires: C, 48.34; H, 4.30; N, 13.26.

***N*-4-Fluorophenylureido-*N*-4-nitrobenzylglycine, E1**: white crystals, mp 194−6 °C; ^{1}H NMR (DMSO-d_6) δ 3.60 (s, 2H, CH_2 of Gly), 4.35 (s, 2H, CH_2 of benzyl), 7.18−7.66 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 7.94 (d, $^3J_{HH}$ = 8.0 Hz, 2H, H_{meta} of FC_6H_4), 8.05 (br s, 2H, NHCONH), 8.22 (d, 2H, H_{meta} of $O_2NC_6H_4$), 11.44 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 40.3 (s, CH_2 of Gly), 44.6 (s, CH_2 of benzyl), 123.8 (s, C_{meta} of $O_2NC_6H_4$), 129.4 (C_{ortho} of $O_2NC_6H_4$), 131.4 (s, C_{meta} of FC_6H_4), 132.0 (s, NH*C*ONH), 135.2 (s, C_{ortho} of FC_6H_4), 144.5 (s, C_{ipso} of $O_2NC_6H_4$), 147.4 (s, C_{para} of $O_2NC_6H_4$), 148.9 (s, C_{ipso} of FC_6H_4), 149.7 (s, C_{para} of FC_6H_4), 177.8 (s, CO_2H). Anal. Found: C, 55.60; H, 4.13; N, 12.00. $C_{16}H_{14}FN_3O_5$ Requires: C, 55.33; H, 4.06; N, 12.10.

***N*-4-Fluorophenylureido-*N*-4-nitrobenzylglycine hydroxamate, F1**: white crystals, mp 201−3 °C; ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 2H, CH_2 of Gly), 4.36 (s, 2H, CH_2 of benzyl), 7.22−7.69 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 7.90 (d, $^3J_{HH}$ = 8.2 Hz, 2H, H_{meta} of FC_6H_4), 8.04 (br s, 2H, NHCONH), 8.24 (d, 2H, H_{meta} of $O_2NC_6H_4$), 8.70 (br s, 1H, N*H*OH), 10.62 (br s, 1H, NHO*H*); ^{13}C NMR (DMSO-d_6) δ 40.1 (s, CH_2 of Gly), 45.4 (s, CH_2 of benzyl), 123.8 (s, C_{meta} of $O_2NC_6H_4$), 129.4 (C_{ortho} of $O_2NC_6H_4$), 130.7 (s, C_{meta} of FC_6H_4), 131.7 (s, NH*C*ONH), 135.5 (s, C_{ortho} of FC_6H_4), 144.7 (s, C_{ipso} of $O_2NC_6H_4$), 145.6 (s, C_{ipso} of FC_6H_4), 147.7 (s, C_{para} of $O_2NC_6H_4$), 148.9 (s, C_{para} of FC_6H_4), 174.8 (s, *C*ONHOH). Anal. Found: C, 52.84; H, 4.32; N, 15.40. $C_{16}H_{15}FN_4O_5$ Requires: C, 53.04; H, 4.17; N, 15.46.

***N*-4-Nitrophenylsulfenyl-*N*-4-nitrobenzylglycine, G1**: yellow crystals, mp 223−4 °C; ^{1}H NMR (DMSO-d_6) δ 3.61 (s, 2H, CH_2 of Gly), 4.37 (s, 2H, CH_2 of benzyl), 6.75 (s, 1H, SNH), 7.21−7.62 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 8.05 (d, $^3J_{HH}$ = 8.3 Hz, 2H, H_{meta} of $O_2NC_6H_4$), 8.21 (d, 2H, H_{meta} of $O_2NC_6H_4$), 11.68 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 40.0 (s, CH_2 of Gly), 44.8 (s, CH_2 of benzyl), 123.8 (s, C_{meta} of $O_2NC_6H_4CH_2$), 129.4 (C_{ortho} of $O_2NC_6H_4CH_2$), 130.3 (s, C_{meta} of $O_2NC_6H_4S$), 135.9 (s, C_{ortho} of $O_2NC_6H_4S$), 144.5 (s, C_{ipso} of $O_2NC_6H_4CH_2$), 145.9 (s, C_{ipso} of $O_2NC_6H_4S$), 147.8 (s, C_{para} of $O_2NC_6H_4CH_2$), 150.4 (s, C_{para} of $O_2NC_6H_4S$), 177.5 (s, CO_2H). Anal. Found: 49.45; H, 3.77; N, 11.24. $C_{15}H_{13}N_3O_6S$ Requires: C, 49.58; H, 3.61; N, 11.56.

***N*-4-Nitrophenylsulfenyl-*N*-4-nitrobenzylglycine hydroxamate, H1**: yellow crystals, mp 182−3 °C; ^{1}H NMR (DMSO-d_6) δ 3.70 (s, 2H, CH_2 of Gly), 4.38 (s, 2H, CH_2 of benzyl), 6.75 (s, 1H, SNH), 7.20−7.67 (m, 4H, H_{ortho} of $CH_3C_6H_4$ + H_{ortho} of $O_2NC_6H_4$), 8.09 (d, $^3J_{HH}$ = 8.2 Hz, 2H, H_{meta} of $O_2NC_6H_4$), 8.23 (d, 2H, H_{meta} of $O_2NC_6H_4$), 8.74 (br s, 1H, N*H*OH), 10.68 (br s, 1H, NHO*H*); ^{13}C NMR (DMSO-d_6) δ, 40.5 (s, CH_2 of Gly), 44.6 (s, CH_2 of benzyl), 123.8 (s, C_{meta} of $O_2NC_6H_4CH_2$), 129.6 (C_{ortho} of $O_2NC_6H_4CH_2$), 130.6 (s, C_{meta} of $O_2NC_6H_4S$), 135.9 (s, C_{ortho} of $O_2NC_6H_4S$), 144.5 (s, C_{ipso} of $O_2NC_6H_4CH_2$), 147.9 (s, C_{para} of $O_2NC_6H_4CH_2$), 148.5 (s, C_{para} of $O_2NC_6H_4S$), 150.3 (s, C_{ipso} of $O_2NC_6H_4S$), 174.6 (s, *C*ONHOH). Anal. Found: C, 47.91, H, 3.65; N, 14.69. $C_{15}H_{14}N_4O_6S$ Requires: C, 47.62, H, 3.73; N, 14.81.

Enzyme Preparations. Human purified MMPs (MMP-1, MMP-2, MMP-8 and MMP-9) were purchased from Calbiochem (Inalco, Milano, Italy). They were activated[26] in the assay buffer by adding bovine trypsin (50 μL, 0.6 mg/mL) to the proenzyme, followed by incubation at 37 °C for 10 min. The trypsin was then inactivated with aprotinin (50 μL, 1.2 mg/mL). Initial rates for the hydrolysis of the thioester substrate AcProLeuGly-S-LeuLeuGlyOEt coupled to the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) were used for assessing the catalytic activity and inhibition of the four MMPs mentioned above, by the spectrophotometric method of Powers and Kam[24a] and Johnson et al.[24b] The change of absorbance (ϵ = 19 800 $M^{-1}\cdot cm^{-1}$)[24a] at 405 nm was monitored continuously at room temperature, using a Cary 3 spectrophotometer interfaced with a PC. A typical 100-μL reaction contained 50 mM MES, pH 6.0, 10 mM $CaCl_2$, 100 μM substrate, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 5 nM MMP. For the K_I determinations, DMSO solutions of the inhibitor were included in the assay, resulting in a final concentration of 2% DMSO in the reaction mixture. In these conditions, K_I values varied from 5% to 10% in replicate experiments. K_I's were then determined by using Easson−Stedman[23] plots and a linear regression program.

C. histolyticum highly purified collagenase and its substrate FALGPA (furanacryloyl-leucyl-glycyl-prolyl-alanine) were purchased from Sigma Chemical Co. (Milano, Italy), and their concentrations were determined from the absorbance at 280 nm and the extinction coefficients furnished by the supplier. The activity of such preparations was in the range of 10 NIH units/mg solid. The potency of standard and newly obtained inhibitors was determined from the inhibition of the enzymatic (amidolytic) activity of the collagenase, at 25 °C, using FALGPA as substrate, by the method of van Wart and Steinbrink.[25] The substrate was reconstituted as 5 mM stock in 50 mM Tricine buffer, 0.4 M NaCl, 10 mM $CaCl_2$, pH 7.50. The rate of hydrolysis was determined from the change in absorbance at 324 nm using an extinction coefficient for FALGPA ϵ_{305} = 24 700 $M^{-1}\cdot cm^{-1}$ in the above-mentioned reaction buffer.[25] Measurements were made using a Cary 3 spectrophotometer interfaced with a PC. Initial velocities were thus estimated using the direct linear plot-based procedure reported by van Wart and Steinbrink.[25] K_I's were then determined according to Easson−Stedman[23] plots and a linear regression program.

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